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Antibodies to TR2 (Herpesvirus Entry Mediator), a New Member of the TNF Receptor Superfamily, Block T Cell Proliferation, Expression of Activation Markers, and Production of Cytokines

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TR2 (TNFR-related 2) is a recently identified member of the TNFR family with homology to TNFRII. We have demonstrated previously that TR2 mRNA is expressed in resting and activated human T cells and that TR2-Ig partially inhibits an allogeneic mixed leukocyte proliferation response. We now characterize TR2 further by the use of specific mAbs. Flow-cytometry analysis using TR2 mAbs confirmed that resting PBL express high levels of cell surface TR2, and that TR2 is widely expressed on all freshly isolated lymphocyte subpopulations. However, stimulation of purified T cells with either PHA or PHA plus PMA resulted in decreased surface expression within 48 h of activation before returning to resting levels at 72 h. TR2 mAbs inhibited $CD4^+$ T cell proliferation in response to stimulation by immobilized CD3 or CD3 plus CD28 mAbs. Assay of culture supernatants by ELISA showed inhibition of TNF-α, IFN-γ, IL-2, and IL-4 production, which, for IL-2 and TNF-α was also confirmed by intracellular cytokine staining. Furthermore, expression of activation markers on $CD4^+$ T cells, including CD25, CD30, CD69, CD71, and OX40 (CD134), was inhibited. TR2 mAbs inhibited proliferation in a three-way MLR, and a response to soluble recall Ag, tetanus toxoid. In conclusion, these results suggest that TR2 is involved in the activation cascade of T cell responses and TR2 mAbs prevent optimal T cell proliferation, cytokine production, and expression of activation markers. *The Journal of Immunology,* **1998, 161: 1786–1794.**

The rapidly expanding nerve growth factor receptor/TNFR superfamily now contains more than 13 members, including TNFRI (1, 2), TNFRII (3),CD27 (4), CD30 (5), CD40 (6, 7), 4-1BB (CDw137) (8), OX40 (9), Fas (10), nerve growth factor receptor (11), lymphotoxin-β receptor (LTBR) (12), Apo-3/DR3/Wsl-1/lymphocyte-associated receptor of death (LARD)/TRAMP (13–17), DR4 (18), DR5/TNF-related apoptosis-inducing ligand (TRAIL)-R2 (19, 20), TRAIL receptor without an intracellular domain (TRID)/DcR1/TRAIL-R3 (19, 21, 22), and OPG[2] (23). A number of viral open reading frames encoding soluble TNFRs have also been identified, including SFV-T2 (3), Va53 (24), G4RG (25), and crmB (26). Although the average amino acid identity between the human receptors ranges from 25 to 35%, receptors in this family share a structural motif in their extracellular domains consisting of three to six cysteine-rich repeats of approximately 30 to 40 amino acids. Sequence identity in the intracellular domains among members of this family is even lower, although TNFRI, Fas, DR3/Wsl-1, DR4, and DR5 receptors encode a conserved death domain (27, 13, 14, 18, 19). Tissue distribution of TNFR superfamily members varies, with ubiquitous expression of TNFRI, TNFRII, and Fas, whereas CD27, CD30, CD40, 4-1BB, and OX40 are mainly restricted to cells of the hemopoietic system (28).

The biologic activities of this family are diverse, such as regulation of cell proliferation, differentiation, cell survival, and cell death (26, 28). A number of superfamily members are involved in the regulation of various aspects of the immune system. For example, CD40, which is expressed predominantly on B lymphocytes, is a costimulatory molecule that plays an important role in B cell activation, Ig class switching, and Ab production (28). Similarly, 4-1BB, CD30, and OX40 expressed on T cells are capable of costimulating T cell activation, cytokine production, proliferation, and differentiation upon binding of their respective ligands.

Our laboratory and others have recently reported a novel member of the TNFR family that has been variously termed TR2 (TNFR-related 2) (29), HVEM (herpesvirus entry mediator) (30, 31), and ATAR (another TRAF-associated receptor) (32). TR2 is expressed mainly in hemopoietic tissues, is high in lymphoid tissues such as spleen and thymus, and is expressed moderately in bone marrow and small intestine. TR2 expression was also detected in purified primary resting and activated CD4- and CD8-positive T cells, and CD19-positive B lymphocytes and monocytes (29). In addition, we showed previously that extracellular TR2 expressed as an Ig fusion protein blocked optimal allogeneic T cell proliferation in a MLR. In this study, we extend the biologic characterization of TR2 and show that mAbs raised to TR2 are capable of inhibiting $CD4^+$ T cell proliferation, IL-2, IFN-γ, IL-4, and

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[2] Abbreviations used in this paper: OPG, osteoprotegerin; HVEM, herpesvirus entry mediator; TR2, tumor necrosis factor receptor-related 2; TRAF, tumor necrosis factor receptor-associated factor; TT, tetanus toxoid.

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Table I. *Affinity of TR2 mAb binding to TR2-Ig*[a]

Ab	Association Constant ($k_{ass}(M^{-1}s^{-1})$)	Dissociation Constant (k_{diss} (s^{-1}))	Affinity (calc. K_D (nM))
12C5	3.6×10^5	1.7×10^{-2}	47
20D4	2.0×10^5	3.4×10^{-4}	1.7
18D4	4.0×10^4	6.0×10^{-3}	152

[a] The affinity of TR2 mAbs, 12C5, 18D4, and 20D4 were determined by immobilizing TR2-Ig on the BIAcore sensor chip followed by binding of TR2 mAbs as described in *Materials and Methods*. Affinity values are expressed as nanomolar concentrations.

Table II. *Binding of TR2 mAbs to hemopoietic cell lines and primary T cells*[a]

Ab	Mean Fluorescent Intensity					
	THP-1	U937	Raji	Jurkat	MG63	$CD4^+$T
TR2 mAb 12C5	25	65	44	33	4	56
Isotype control (MsIgG1)	8	13	27	5	4	16
Secondary control (GAM-FITC)	9	3	3	6	4	3

[a] Murine TR2 mAb, 12C5, was used to detect TR2 expression on lymphoid, myeloid, and primary T cells. 12C5 binding was detected using FITC-labeled goat anti-mouse IgG. Isotype (GAM-FITC) and control mouse IgG1 mAb was included to detect nonspecific binding. Cell lines tested were THP-1 and U937 (myeloid cell lines), Raji (B cell line), Jurkat (T cell line), and MG63 (osteosarcoma cell line). Samples were analyzed on a BD FACSort using CellQuest software. Results are expressed as mean fluorescent intensity. Data are representative of three experiments.

TNF-α secretion and cell surface receptor expression, indicating that TR2 is involved in the control of optimal T lymphocyte activation.

Materials and Methods

Antibodies

Directly conjugated Abs CD27, CD30, CD69, CD71, CD40L (CD154) (PharMingen, San Diego, CA) OX40 (Becton Dickinson Immunocytometry Systems, San Jose, CA), CD54, CD58, and CD11a (Immunotech, Westbrook, ME) were obtained commercially. Control D12 mAb to $\alpha_v\beta_3$ was supplied by Dr. Z. Jonak (SmithKline Beecham Pharmaceuticals, King of Prussia, PA).

Production of rTR2-Ig fusion protein

PCR primers were designed to clone the region of the TR2 cDNA encoding the extracellular domain with a 5′ *Eco*RI and *Bgl*II site, and a 3′ factor Xa protease and Asp718I site (5′-cag gaa ttc gca gcc atg gag cct cct gga gac tg-3′ and 5′-cca tac cca ggt acc cct tcc ctc gat aga tct tgc ctt cgt cac cag cca gc-3′). The PCR product was digested with *Eco*RI and Asp^{718I} and ligated into the COSFclink plasmid (33) to produce TR2Fclink. This vector encodes amino acids 1–192 of TR2, followed by the amino acids RSIEGRGT for factor Xa cleavage, and residues 226–458 of human IgG1.

TR2Fclink was transfected into COS cells for transient expression, or Chinese hamster ovary cells to produce stable cell lines after selection in nucleotide-free medium. TR2-Ig protein was purified from supernatants by protein G chromatography (Pharmacia LKB Biotech, Piscataway, NJ). Soluble TR2 was generated by incubation of TR2-Ig with factor Xa (Bio Labs, Beverly, MA) at 4°C overnight at a ratio of 1:200, factor Xa:TR2-Ig. TR2 and Ig were separated by protein G chromatography, and soluble TR2 was used for immunization of mice for mAb production.

mAb production

Mice (F_1 hybrids of BALB/c and C57BL/6) were immunized s.c. with 10 μg of TR2 in CFA, and 4 wk later with 10 μg of TR2 in IFA. On the basis of a good serum Ab titer to TR2, one mouse received further immunizations of 8 μg of TR2 (i.p. in saline) at 8 wk, and 2 days later. Two days following the final immunization, a splenectomy was performed. Mouse spleen cells were used to prepare hybridomas by standard procedures. Positive hybridomas were cloned by limiting dilution methods. Hybridoma supernatants were tested for binding in 96-well plates coated with TR2-Ig at 0.25 μg/ml and detected using conjugated anti-mouse Ab. Positive hybridomas were scaled up and mAbs were purified by ProsepA (Bio Processing, Consett, Durham, U.K.) chromatography, respectively, using the manufacturer's instructions. mAbs were >95% pure by SDS-PAGE.

Affinity analysis

Surface plasmon resonance (BIAcore, Uppsala, Sweden) was conducted with TR2-Ig immobilized to the sensor chip surface and a flow rate of 10 μl/min with HEPES-buffered saline (34). The mAb was first bound to the TR2-Ig (approximately 500 response units), followed by injections of TR2 (0–20 μg/ml, 30 μl), buffer flow for 600 s, and regeneration of the sensor chip surface with 100 mM phosphoric acid. A log n (dR/dt) vs t plot was used for association phase analysis and Ln (R1/Rn) vs t plot for dissociation phase analysis utilizing BIAcore software.

Purification of primary cells

PBMCs from volunteer donors were purified by Ficoll-Hypaque density-gradient centrifugation (Pharmacia LKB Biotech), washed, and resuspended in RPMI 1640 medium supplemented with 10% FBS, 25 mM HEPES buffer, 2 mM L-glutamine, and 50 μg/ml gentamicin. Purified $CD4^+$ T lymphocytes were obtained by elution through a T cell column (R&D Systems, Minneapolis, MI) and negative selection using immunomagnetic $CD8^+$ Dynabeads (Dynal, Lake Success, NY). Purity was routinely >95% $CD4^+$ cells determined by flow cytometry.

Flow cytometry

All surface staining was conducted using staining buffer consisting of PBS supplemented with 0.2% BSA and 0.1% sodium azide. Cells were preincubated with unconjugated goat or mouse IgG Ab for 10 min on ice to block nonspecific binding of conjugated Abs. Cells were incubated with primary unlabeled or biotinylated mAb for 30 min at 4°C, washed twice, and incubated for another 30 min with conjugated secondary Ab or streptavidin-phycoerythrin (Sigma, St. Louis, MO) at 4°C. Cells were washed three times, fixed in 2% formaldehyde solution in PBS, and analyzed using a BD FACSort. Data were analyzed using CellQuest software (Becton Dickinson, San Jose, CA).

For intracellular cytokine staining, cells were surface stained as above before fixation in 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA). After washing, cells were blocked with purified mouse or rat IgG in PBS containing 1% normal mouse serum, 0.1% saponin, and 0.1% sodium azide for 10 min on ice. Conjugated anti-human cytokine mAbs were incubated with permeabilized cells for 30 min at 4°C; washed twice in PBS containing 1% NMS, 0.1% sodium azide, and 0.01% saponin; and washed once in PBS with BSA and azide. Samples were analyzed by flow cytometry, as described above.

Anti-CD3 and anti-CD3/CD28 stimulation

Flat-bottom 96-well microtiter plates or petri dishes (Falcon, Franklin Lakes, NJ) were coated with 10 μg/ml goat anti-mouse IgG (Sigma) in PBS containing 1% FBS at 4°C overnight. After three washes, CD3 mAb was incubated in the plates for 2 h at 37°C and washed three times, and purified $CD4^+$ T cells were added at 10^6 cells/ml together with a 1/500 dilution of CD28 mAb ascites fluid. Supernatants for ELISA detection of secreted cytokines were taken at 48 h. For proliferation, cultures were incubated at 37°C for 72 h. [methyl^{-3}H]Thymidine was added to cultures for the last 6 h, plates were harvested (Skatron, Sterling, VA), and thymidine incorporation was determined using a Wallac β-plate scintillation counter (Wallac, Gaithersburg, MD).

Three-way MLR

PBMCs from volunteer donors were purified by density-gradient centrifugation (Pharmacia LKB Biotech). PBMCs from two donors were adjusted to 1×10^6 cells/ml in RPMI 1640 medium (Life Technologies, Grand Island, NY), supplemented with 10% FCS, 2 mM L-glutamine, 50 μg/ml gentamicin, and 25 mM HEPES buffer. PBMCs from a third donor were adjusted to 2×10^5 cells/ml. Fifty microliters of PBMCs from each donor were added to wells of a 96-well round-bottom microtiter plate (Falcon). Dilutions of mAb were added in quadruplicate to microtiter wells. Cells were cultured for 6 days at 37°C in 5% CO_2, and 1 μCi of [^{3}H]thymidine was added to wells for the last 6 h of culture. Cells were harvested as described above.

Tetanus toxoid recall assay

Donors were prescreened for recall responsiveness to tetanus toxoid (TT). PBMCs were cultured at 1×10^6 cells/ml in AIM V medium (Life Technologies, Gaithersburg, MD) containing 10% autologous serum, 25 mM

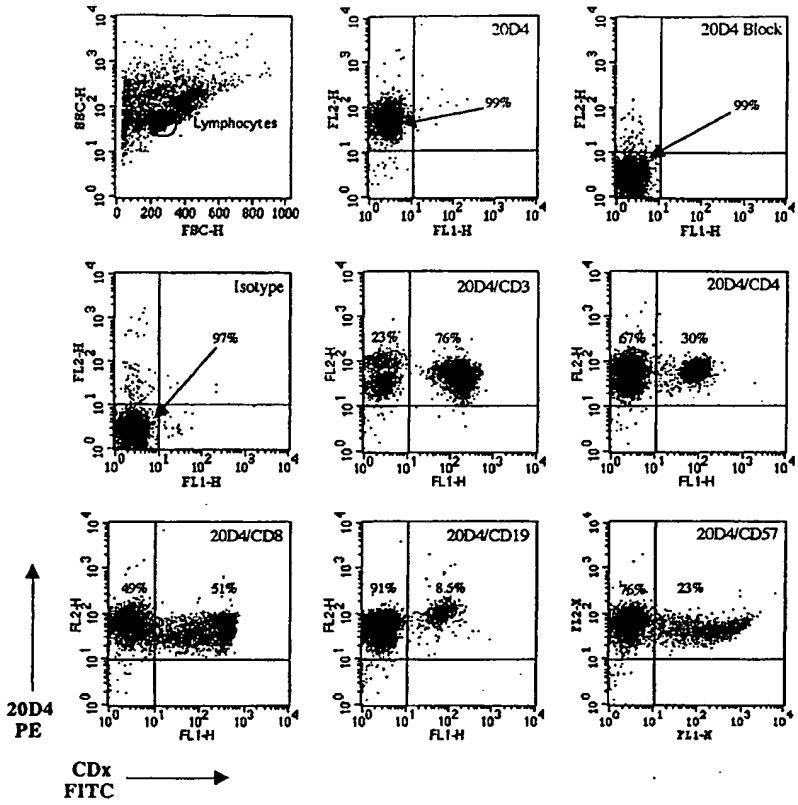

FIGURE 1. Expression of cell surface TR2 on PBMC subpopulations. PBMCs were prepared as described previously, and were stained using biotinylated TR2 mAb 20D4 in combination with mAbs to different CD markers. Specificity of 20D4 binding was determined by preincubating cells with a 10-fold excess (block) of nonbiotinylated 20D4 mAb before the addition of biotinylated 20D4 mAb. Samples were analyzed on a BD FACSort using CellQuest software. Quadrants were set using isotype controls, and percentage of positive cells is indicated in each quandrant. Data represent one of three experiments.

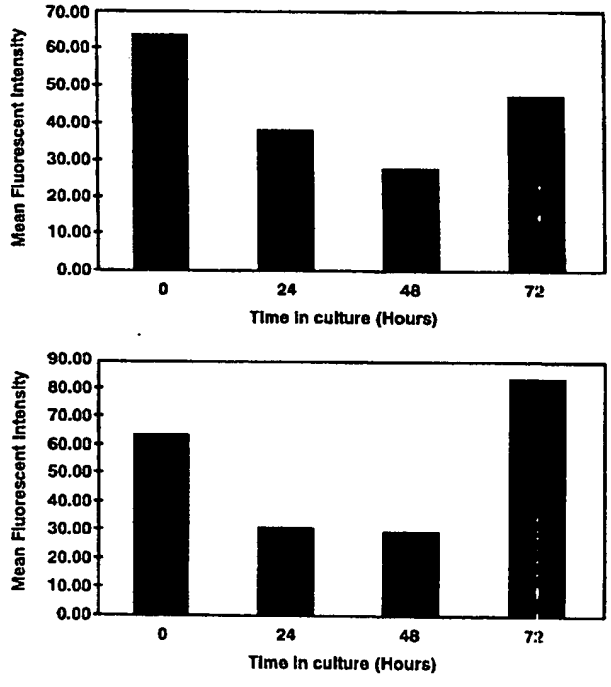

FIGURE 2. Kinetic expression of TR2 on purified $CD4^+$ T cells following activation with PHA or PHA plus PMA. Purified $CD4^+$ T cells were stimulated with PHA (*top panel*) or PHA plus PMA (*bottom panel*) for 72 h. Cells were surface stained using TR2 mAb 12C5, followed by goat anti-mouse FITC-conjugated Ab. Data were analyzed on a BD FACSort and using Cellquest software. Data presented are an example of two similar experiments.

HEPES buffer, 2 mM L-glutamine, and 50 μg/ml gentamicin in the presence of 1/800 dilution of TT (Massachusetts Public Health Biologic Laboratory, Jamaica Plain, MA) in 96-well round-bottom microtiter plates. Proliferation was monitored after 5 days following the addition of 1 μCi of [^{3}H]thymidine for the last 6 h of culture, as described previously.

Results

Affinities of TR2 mAbs

Three murine mAbs to TR2 were generated and their affinities were measured by surface plasmon resonance on a BIAcore instrument. The affinity and kinetic data for 12C5, 18D4, and 20D4

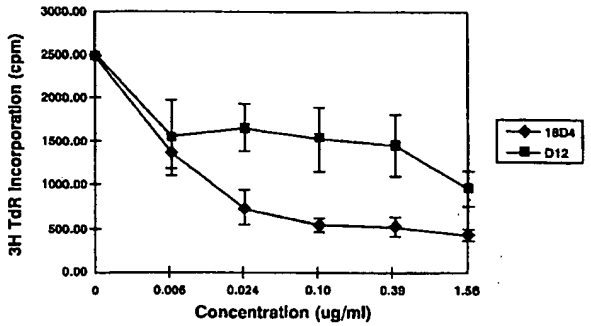

FIGURE 3. Effect of TR2 mAbs on CD3-stimulated $CD4^+$ T lymphocyte proliferation. Purified primary $CD4^+$ T lymphocytes were stimulated in 96-well flat-bottom plates precoated with 5 μg/ml of CD3 mAb in the presence of either TR2 (◆) or control mAb (■) for 72 h. Proliferation was monitored by [^{3}H]thymidine incorporation during the last 6 h of culture, and cells were harvested for β-scintillation counting. Data represent one of three similar experiments.

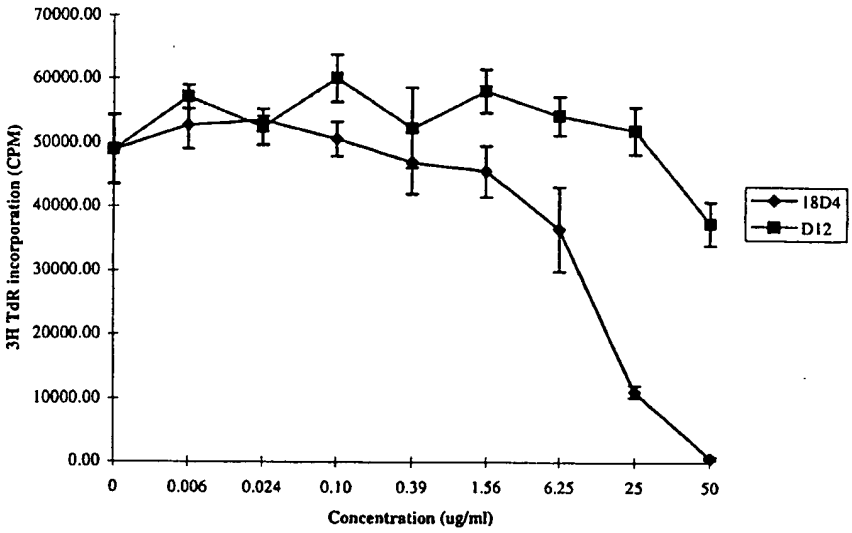

FIGURE 4. Effect of TR2 mAb on CD3- and CD28-stimulated $CD4^+$ T lymphocyte proliferation. Purified primary $CD4^+$ T lymphocytes were stimulated in 96-well flat-bottom plates precoated with 5 μg/ml of CD3 mAb in the presence of CD28 mAb with either TR2 (◆) or control mAb (■) for 72 h. Proliferation was monitored by [^{3}H]thymidine incorporation during the last 6 h of culture, and cells were harvested for β-scintillation counting. Data represent one of three similar experiments.

are shown in Table I. Affinities for 12C5, 18D4, and 20D4 were 47, 152, and 1.7 nM, respectively, which is within the affinity range seen for mAbs to other members of this family. TR2 mAbs showed no binding to other members of this superfamily, such as OPG-Ig and DR3-Ig, by surface plasmon resonance. Blocking experiments using these mAbs indicated that 12C5 and 18D4 bound to overlapping epitopes on the TR2 receptor, whereas 20D4 bound to an epitope distinct from 12C5 and 18D4 (data not shown). In addition, the specificity of TR2 mAbs for TR2 and other closely related receptors was tested by ELISA. TR2 mAbs showed no binding to soluble TNFR 1, soluble TNFR 2, CD40-Ig, OPG-Ig, or DR3-Ig (data not shown).

Cell surface expression of TR2 on cell lines and primary T lymphocytes

Previously, we demonstrated that hemopoietic derived cell lines express mRNA for TR2 and that, following activation, TR2 mRNA levels were maintained (29). T and B lymphocyte and monocyte/macrophage-derived cell lines positive for TR2 mRNA were used to demonstrate whether mAbs generated to TR2 were capable of recognizing native cell surface-expressed receptor. Using TR2 mAb 12C5, cell surface TR2 was detected on Jurkat (T cell), U937, THP-1 (myeloid), and Raji cells (B cell), but not MG63 cells (osteosarcoma line), consistent with the mRNA expression data (Table II). Similarly, freshly isolated peripheral blood $CD4^+$ T cells also expressed high levels of surface TR2 and TR2 mRNA (29).

PBMC subpopulations were examined for TR2 expression by two-color flow cytometry using TR2 mAb 20D4 in combination with mAbs to different cell surface markers (Fig. 1). TR2 was expressed on all lymphoid subpopulations, including $CD3^+$ (T), $CD4^+$ and $CD8^+$ (T subpopulations), $CD19^+$ (B cells), and $CD57^+$ (NK cells). This result was expected, as we have shown previously that purified peripheral lymphocyte subpopulations express high levels of TR2 mRNA (29).

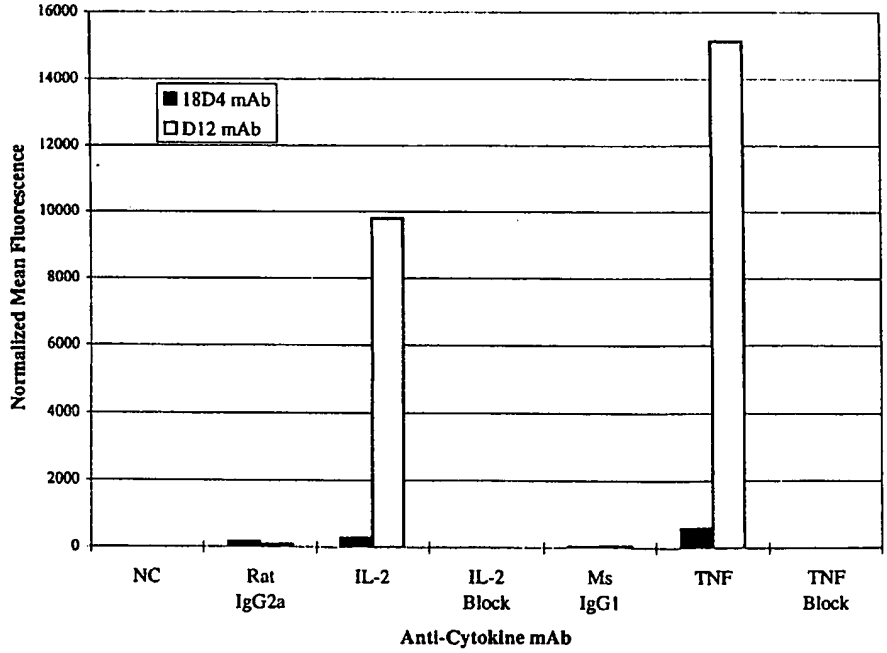

FIGURE 5. Inhibition of $CD4^+$ T lymphocyte IL-2 and TNF-α production by TR2 mAb. Purified primary $CD4^+$ T lymphocytes were stimulated with CD3 and CD28 mAbs in the presence of 25 μg/ml of TR2 mAb 18D4 (■) or control D12 mAb (□) for 14 h with brefeldin A. Conjugated isotype control mAbs were used as controls. Blocks were used to determine the specificity of anti-cytokine mAbs by preincubating anti-cytokine mAbs with a 10-fold molar excess of human rIL-2 or human rTNF-α before the addition to permeabilized cells. Cells were assessed for intracellular cytokine production, as described in *Materials and Methods*. Similar results were obtained in a second experiment.

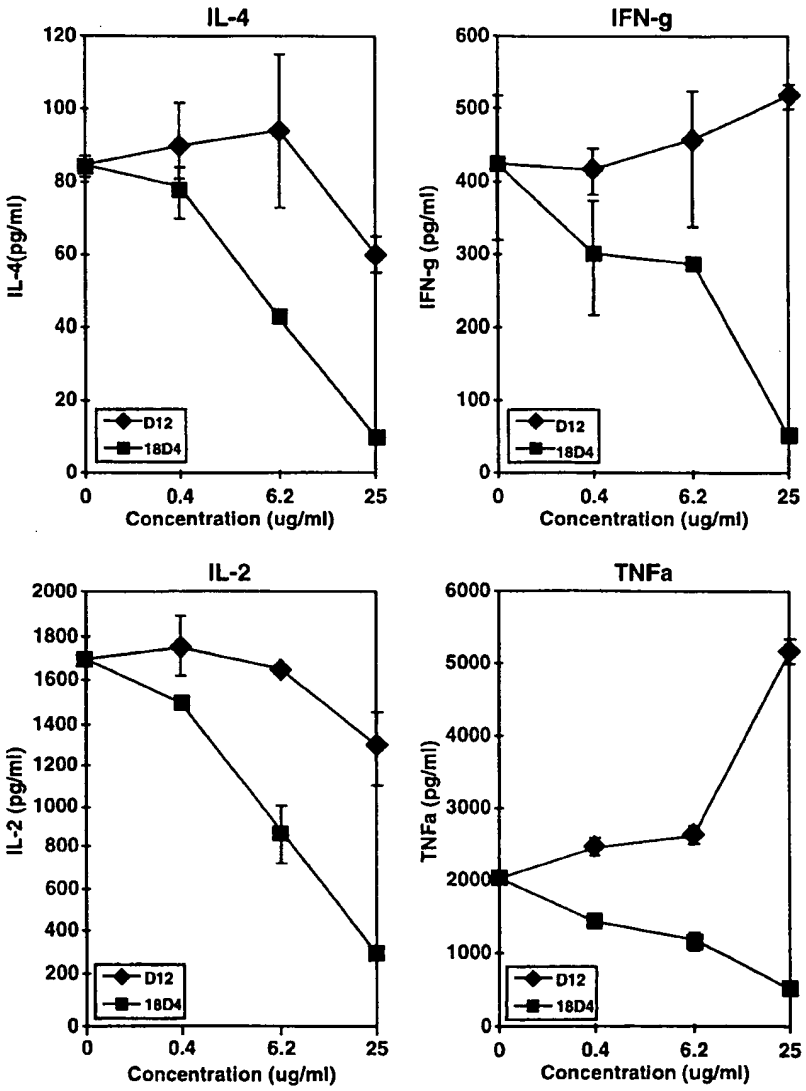

FIGURE 6. Effect of TR2 mAb 18D4 on $CD4^+$ T lymphocyte cytokine secretion. Purified $CD4^+$ T lymphocytes were stimulated with immobilized anti-CD3 and anti-CD28 mAbs in the presence of 25 μg/ml of either mAb 18D4 (■) or control mAb D12 (◆). Culture supernatant was removed and frozen at −20°C and assayed using ELISA kits for individual cytokines (R&D Systems).

Next we examined the kinetics of TR2 expression on primary $CD4^+$ T lymphocytes following activation. Resting or activated $CD4^+$ T cells were monitored for TR2 expression using 12C5 over a 72-h period (Fig. 2). High levels of TR2 were detected on resting lymphocytes, but after 24 h of PHA stimulation, surface expression of TR2 declined. This was maintained for 72 h, after which TR2 expression increased. A similar pattern was observed following combined PHA and PMA stimulation. Similar data were obtained using TR2 mAbs 12C5 and 18D4 (unpublished data).

Inhibition of $CD4^+$ T lymphocyte proliferation

Since TR2 appeared to be expressed on both resting and activated $CD4^+$ T lymphocytes, this suggested that TR2 may be involved in T cell growth and differentiation, similar to other members of this family, such as CD27, OX40, and 4-1BB. Purified $CD4^+$ T lymphocytes were stimulated with immobilized CD3 mAb alone or in combination with CD28 mAb in the presence of TR2 or control mAbs in a 72-h proliferation assay (Figs. 3 and 4). Potent inhibition of CD3-induced proliferation was seen with 18D4 from 24 ng/ml to 1.5 μg/ml (Fig. 3). A control mAb, D12, failed to inhibit proliferation at the same concentrations. CD3 plus CD28 costimulation was also inhibited by 18D4 mAb, albeit at higher concentrations, with inhibition being detected above 6 μg/ml (Fig. 4). No inhibition was detected with control mAb. Forward and side scatter profiles by FACS indicated that T cells treated with TR2 mAb were capable of blastogenesis and the numbers of blast cells were similar to that seen with control D12 mAb (unpublished data). In addition, apoptosis in D12- and 18D4-treated cell cultures was examined by *T*dT-mediated d*U*TP nick-end labeling and annexin V staining. No significant numbers of apoptotic cells could be detected in either population of cells 18 h after stimulation. These data indicate that TR2 is involved in $CD4^+$ T lymphocyte proliferation in response to CD3 or CD3 plus CD28 stimulation.

Inhibition of $CD4^+$ T lymphocyte cytokine production

Since T cell proliferation appeared to be regulated in part by TR2, we determined the effect of TR2 on cytokine production. $CD4^+$ T lymphocyte cytokine production was examined by intracellular cytokine staining using flow cytometry and by ELISA from cell culture supernatants. CD3 and CD28 mAb-stimulated $CD4^+$ T lymphocytes were cultured for 14 h with either 25 μg/ml TR2 mAb 18D4 or control mAb D12 in the presence of the Golgi transport inhibitor, brefeldin A. As shown in Figure 5, TR2 mAbs completely inhibited both TNF-α and IL-2 intracellular production,

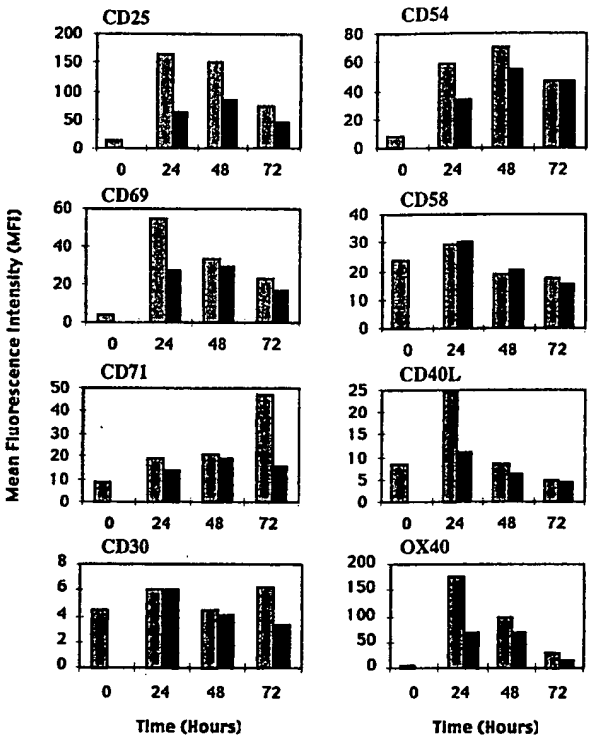

FIGURE 7. Modulation of $CD4^+$ T lymphocyte receptor expression by TR2 mAbs following CD3/CD28 costimulation. Purified $CD4^+$ T lymphocytes were stimulated with immobilized anti-CD3 (5 μg/ml) and anti-CD28 mAb in the presence of either 25 μg/ml of TR2 mAb 18D4 (solid) or control D12 mAb (grey). Cell surface expression of CD25, CD71, CD69, CD30, CD54, CD58, OX40, and CD40L was monitored from 0 to 72 h by flow cytometry using directly conjugated mAbs. Data are expressed as mean fluorescent units, and are representative of three similar experiments.

whereas high levels of both cytokines were detected in cells treated with control D12 mAb. The specificity of anticytokine binding was confirmed by preincubating conjugated anti-cytokine mAbs with 10-fold molar excess of recombinant TNF-α or IL-2.

In a separate experiment, 48-h culture supernatants from CD3 and CD28 mAb-stimulated cells were harvested and assayed for cytokine levels by ELISA (Fig. 6). Treatment of stimulated $CD4^+$ T lymphocytes with TR2 mAb inhibited IL-2, TNF-α, IL-4, and IFN-γ secretion in a dose-dependent manner, whereas cells incubated with a control mAb secreted similar levels of cytokines produced by cells stimulated in medium alone. TR2 mAb 18D4 and control mAb D12 were not found to inhibit the detection of cytokines by ELISA, indicating that TR2 mAb did not inhibit detection of cytokine by ELISA (unpublished data).

Cell receptor expression and morphology

Expression of receptors on T cells during stimulation has been shown to modulate the capacity of T cells to respond to activation signals. As TR2 mAbs down-regulated T cell proliferation and cytokine production, we also determined whether they were capable of modulating cell surface receptor expression (Fig. 7). At various times, TR2 mAbs suppressed the expression of the proliferation-associated marker CD71 (transferrin receptor) and CD25 (IL-2Rα), the early activation marker CD69, the costimulatory receptors CD30 and OX40, and the B cell stimulatory CD40 ligand. However, no effect was observed on the surface expression of CD27 (unpublished data). Furthermore, the adhesion molecule CD54 (ICAM-1), but not CD58 (LFA-3) or CD11a (LFA-1; unpublished data), was inhibited by TR2 mAb. This was reflected in the reduced size of lymphocyte aggregates in cultures stimulated in the presence of TR2 mAb (Fig. 8) compared with the larger cell aggregates seen in cultures treated with control mAb. Together these data indicate that TR2 is involved in controlling receptor expression on activated $CD4^+$ T cells.

Inhibition of Ag-specific proliferative responses to insoluble and soluble Ags

Having established that TR2 mAbs were capable of inhibiting T cell mitogenesis, cytokines, and surface receptors, we investigated the effect of TR2 mAbs on Ag-specific allogeneic responses in a three-way MLR. TR2 mAb 12C5 inhibited proliferation in a dose-dependent manner from 1.5 to 100 μg/ml, whereas 18D4 only blocked proliferation at the highest concentrations (Fig. 9). In contrast, CD4 mAb inhibited proliferation at all concentrations tested, with maximal inhibition of 94% at 1 μg/ml. Control IL-5 mAb failed to affect MLR proliferation at all concentrations tested.

The effect of TR2 mAbs on Ag-specific proliferative responses to soluble Ag was also determined. Memory recall proliferation responses to TT were inhibited by TR2 mAb 18D4 from 25 to 50 μg/ml, whereas proliferation in the presence of control mAb had no effect (Fig. 10). This indicates that TR2 is involved in optimal Ag-specific responses to both insoluble and soluble Ags, and is involved in memory T cell responses to TT.

Discussion

In the present study, we have further characterized the function of TR2/HVEM using specific mAbs and confirmed its role in T cell biology. We have shown that TR2 mAbs abrogate T cells function by 1) inhibiting cytokine production, 2) inhibiting cytokine receptor expression, 3) decreasing the expression of costimulatory ligands and receptors, and 4) inhibiting the expression of adhesion molecules. This suggests that TR2 signaling is upstream of these events and is involved in the early stages of T cell activation and differentiation.

We first examined the distribution of TR2 on PBL. TR2 is expressed on T cells as well as on the majority of B lymphocytes and NK cells, which is consistent with the distribution of TR2 mRNA (29). This indicates that TR2 is widely expressed on resting PBL and is not restricted to any subpopulations, a distribution that is unique to this superfamily. The wide distribution of TR2 has been confirmed using the recently identified ligand for TR2 (61) (Harrop et al., submitted) in FACS-binding experiments that showed a similar binding pattern to TR2 mAbs (J.A.H., manuscript in preparation). TR2 was also detected on the majority of naive ($CD45RA^+$) and memory ($CD45RO^+$) lymphocytes (data not shown). Activation of $CD4^+$ T lymphocytes with PHA or PHA plus PMA resulted in a temporary reduction in surface TR2 expression. Northern blot data indicated that TR2 mRNA increased slightly following activation with PHA and PMA, suggesting that TR2 expression is not controlled at the mRNA level (29).

Inducible and constitutive expression of TNFR superfamily members has been reported on primary T lymphocytes. Detectable expression of TNFRII (35), CD30 (36), 4-1BB, CD95, and OX40 (37–39) is generally observed after activation of T lymphocytes. However, CD27 is expressed constitutively on resting $CD4^+$ T cells, but increases after activation (40). Cleavage of CD27 from $CD45RO^+$ memory T cells occurs following repeated stimulation, and has been used as a marker of chronically activated memory cells (41, 42). Interaction of CD27 with its ligand (CD70) results in the down-regulation of cell surface CD27 and an increase in soluble CD27 (43).

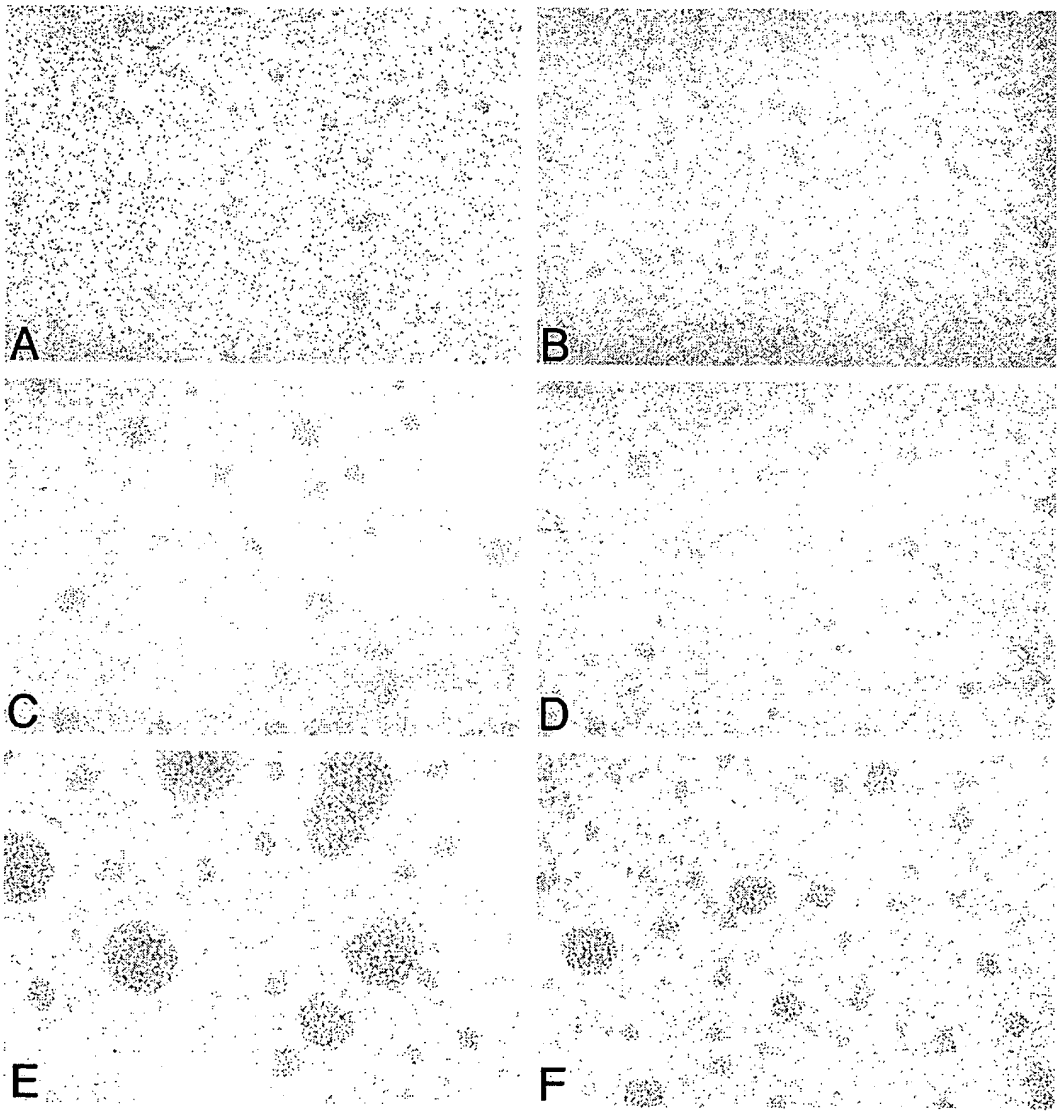

FIGURE 8. Effect of TR2 mAb on the morphology of $CD4^+$ T lymphocytes after activation with CD3 plus CD28 mAbs. Purified peripheral $CD4^+$ T lymphocytes were stimulated with immobilized CD3 plus CD28 mAbs in the presence of either control mAb D12 (25 μg/ml; *A*, *C*, *E*) or TR2 mAb 18D4 (25 μg/ml; *B*, *D*, *F*). Photographs were taken after 24 (*A*, *B*), 48 (*C*, *D*), and 72 h (*E*, *F*) at ×40 magnification. Data are representative of three experiments.

Similar mechanisms may be involved in the down-regulation of TR2 on activated T lymphocytes, as TR2-ligand is expressed on activated T cells and soluble TR2 has been demonstrated in patient samples (J.A.H., unpublished observation).

Members of the TNFR superfamily have been shown to be involved in activation and differentiation of the immune system, including the regulation of proliferation, cytokine production, receptor expression, and cell survival. TR2 mAbs inhibited both suboptimal and optimal $CD4^+$ T lymphocyte proliferation in response to CD3 mAb alone or CD3 plus CD28 mAb, respectively. Inhibition of proliferation during CD3 plus CD28 stimulation also resulted in abrogation of IL-2, IL-4, TNF-α, and IFN-γ production. Since IL-2Rα expression was also inhibited by TR2 mAbs, this suggests that decreased proliferation could in part be attributed to inhibition of the IL-2 pathway. Furthermore, the inhibition of TNF-α and IFN-γ suggests that TR2 contributes to proinflammatory cytokine production by T lymphocytes. Abrogation of IL-2 and TNF-α production was also seen at the single cell level using intracellular cytokine staining, indicating that reduced cytokine production was not due to reduced cell numbers as a result of decreased proliferation following TR2 mAb treatment.

Interestingly, other members of the TNFR superfamily were down-regulated by TR2 mAb treatment, including CD30 at 72-h and OX40 at 24-h stimulation, both of which have been shown to be costimulatory molecules for T cell activation (44, 45), and to provide help for B cell activation and differentiation (46, 36). This indicates that TR2 is upstream of CD30 and OX40 in the T cell activation cascade.

Selective regulation of adhesion molecules was also observed with reduced surface expression of CD54 (ICAM-1), but not its ligand CD11a (LFA-1) (J.A.H., unpublished data) or CD58 (LFA-3). Inhibition of LFA-1/ICAM-1 function has been shown previously to directly inhibit lymphocyte aggregation (47, 48), and can result in suboptimal responses as a result of reduced cell to cell conjugation (49). Consistent with this, homotypic aggregates of activated T lymphocyte blasts were smaller in cultures incubated with 18D4 compared with control mAb, which may result from a combination of reduced adhesion receptor expression such as ICAM-1 expression and decreased proliferation seen following TR2 mAb treatment.

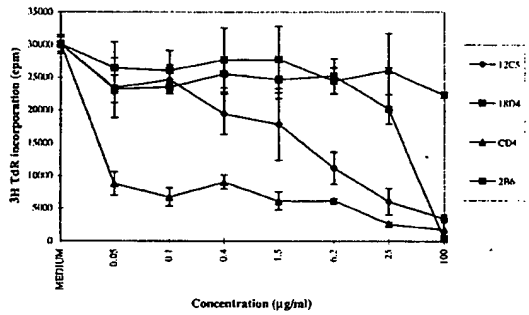

FIGURE 9. Inhibition of three-way MLR proliferation responses by TR2 mAbs. The capacity of TR2 mAbs, 12C5 (●) and 18D4 (■), to inhibit allogeneic proliferation was examined in a three-way MLR. Anti-CD4 mAb (▲) and 2B6 anti-human IL-5 mAb (◆) were included as controls. PBMCs from three donors were incubated for 6 days with mAbs, cells were labeled with [^{3}H]thymidine for the last 6 h of culture, and incorporation was assessed using a β-scintillation counter. Experiment represents one of three similar assays.

Ag-specific immune responses were also inhibited by TR2 mAbs, including MLR proliferation and TT recall responses, suggesting that TR2 is involved in Ag-specific responses to both insoluble and soluble Ags. TR2 mAbs may also affect non-T cells. Expression of TR2 mRNA has been demonstrated in cells with Ag-presenting function such as monocytes/macrophages and B lymphocytes (29). Hence, TR2 mAbs may be inhibiting Ag-specific T cell proliferative responses by disrupting APC functions, such as costimulatory molecule expression (CD40, CD80, CD86, HLA-DR) and/or cytokine production. The effect of TR2 mAbs on monocyte and B cell function needs to be addressed to determine whether signals delivered by TR2 ligand(s) are involved in APC function.

T cell stimulation has previously been shown to involve other members of the TNFR superfamily. Blockade of 4-1BB/4-1BBL inhibited murine splenocyte responses to soluble CD3 and allogeneic proliferation, whereas 4-1BB mAbs or cells transfected with 4-1BBL induced strong proliferative responses in primary human T cells costimulated with mitogen (50–53). Costimulation of T lymphocytes with CD30L or CD30 mAbs enhanced proliferative responses and cytokine production (36, 45, 54). Exceptions to this include TNFRI and Fas, in which agonist mAbs initiate programmed cell death (55, 56, 14). Hence, TR2 mAbs reported in this study are unusual since they appear to act as antagonists rather than agonists. However, inhibitory mAbs have been reported for members of this family without death domains. CD27 mAbs have been shown to block soluble Ag- and mitogen-stimulated T cell proliferation and PWM-driven B cell proliferation (57, 58). Abs to CD40 that inhibit the production of Ig by B cells have also been reported (59).

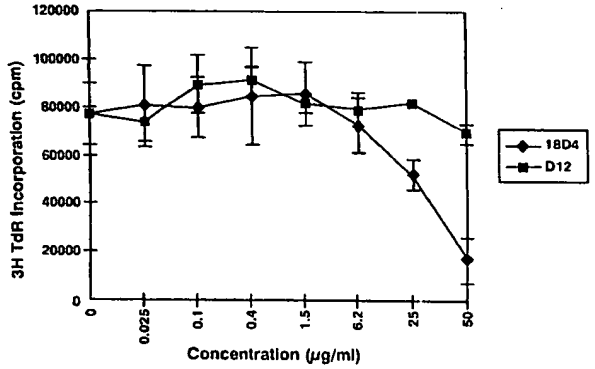

FIGURE 10. Inhibition of PBMC recall response to TT. PBMCs from a TT-responsive donor were cultured with 1/800 dilution of TT in the presence of TR2 (◆) or control mAb (■) for 5 days. Cells were labeled with [^{3}H]thymidine for the last 6 h of culture, and incorporation was assessed using a β-scintillation counter, as described previously. Experiment represents one of three similar assays.

Differences in the potency of TR2 mAbs 18D4 and 12C5 were observed in T cell proliferation assays described above. Both mAbs have similar affinities for TR2 and bind to overlapping epitopes. Hence, differences in activities of these mAbs may reflect either the capacity to block ligand binding or to act as functional antagonists. We and others have since identified one of the ligands for TR2, called LIGHT or HVEM-L (61) (J.A.H., submitted). TR2 mAbs 12C5 and 18D4 can block HVEM-L binding to TR2 in FACS and ELISA assays. Consistent with this, TR2-Ig shows similar activity to TR2 mAbs in Ag-specific proliferation assays (J.A.H., unpublished data). TR2 appears to be involved in the T cell activation cascade, which is consistent with previous reports on TR2 signaling. TR2 encodes a short cytoplasmic tail (29) that does not contain a death domain seen with TNFRI, Fas, DR3, DR4, and DR5 receptors (26, 15, 18, 19). The cytoplasmic region of TR2 has a 9-amino-acid region previously found to be crucial in TRAF binding to the cytoplasmic domains of TNFRII, CD30, and CD40 (60). Overexpression of TR2 in HEK 293 cells stimulates TRAF 1, 2, 3, and 5 binding, activation of NF-κB, Jun N-terminal kinase, and AP-1 (31, 32). NF-κB and AP-1 signaling stimulates cellular activation, whereas we show in this study that TR2 mAbs inhibit T cell activation, indicating that TR2 mAbs block the interaction of TR2 with its ligand(s) and prevent activation of T cells. Alternatively, TR2 may exist as a heteromeric receptor with another member of the superfamily, leading to the transduction of signals different from TR2 homotrimers. The mechanism by which TR2 mAbs block T cell activation and their capacity to stimulate NF-κB signaling are currently under investigation in our laboratory. Further characterization of the interaction between LIGHT/HVEM-L and TR2 will help to further define the importance of this receptor in T lymphocyte activation and differentiation.

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